Aldosterone Potentiates Angiotensin II–Induced Signaling in Vascular Smooth Muscle Cells

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Background—In a double-transgenic human renin and human angiotensinogen rat model, we found that mineralocorticoid receptor (MR) blockade ameliorated angiotensin II (Ang II)–induced renal and cardiac damage. How Ang II and aldosterone (Ald) might interact is ill defined.

Methods and Results—We investigated the effects of Ang II (10^{-7} mol/L) and Ald (10^{-7} mol/L) on extracellular signal–regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signaling in vascular smooth muscle cells (VSMCs) with Western blotting and confocal microscopy. Ang II induced ERK 1/2 and JNK phosphorylation by 2 minutes. Ald achieved the same at 10 minutes. Ang II + Ald had a potentiating effect by 2 minutes. Two oxygen radical scavengers and the epidermal growth factor receptor (EGFR) antagonist AG1478 reduced Ang II–, Ald–, and combination-induced ERK1/2 phosphorylation. Preincubating the cells with the MR blocker spironolactone (10^{-6} mol/L) abolished Ang II–induced ROS generation, EGFR transactivation, and ERK1/2 phosphorylation.

Conclusions—Ald potentiates Ang II–induced ERK-1/2 and JNK phosphorylation. Oxygen radicals, the MR, and the EGFR play a role in early signaling induced by Ang II and Ald in VSMCs. These in vitro data may help explain the effects of MR blockade on Ang II–induced end-organ damage in vivo. (Circulation. 2004;109:2792-2800.)

Key Words: angiotensin • aldosterone • receptors • kinases • reactive oxygen species
light ether anesthesia. Three measurements per heart were averaged. Rats were killed at the age of 7 weeks.

Cell Culture
Aortic VSMCs were isolated from SD rats.12 Passages 2 to 4 were used for immunohistochemistry and passage 4 to 10 for Western blotting. VSMCs were phenotyped by staining for muscle-specific α-actin (Dako) and desmin (Boehringer-Mannheim). VSMCs were also analyzed for MR expression. TaqMan polymerase chain reaction (PCR) demonstrated RNA expression of the receptor (primer sequences as follows: MR-F, GCATCACACCATCCCCG; MR-R, TCTGAGGCTGACATAACGGTC; MR-P, FAM-CATGGCCT-GAGAATCCTCAGGCT-TAMRA; data not shown). Cells were treated with Ang II (Sigma), Ald (Clinalfa), glutathione (GSH) or Tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid; Sigma), or Spi (Sigma). All experiments were performed under 18-hour serum-free conditions.

Immunohistochemistry
Confocal microscopy was performed as described.12 At least 50 to 80 cells from each experiment were examined under each condition by 2 different investigators without knowledge of the origin of the specimens. Quantification was performed with histogram function in the MRC Laser Sharp software. The subcellular regions were outlined manually, and the calculated mean fluorescence intensity was obtained for the delineated regions. Data are presented as the mean fluorescence intensity in the respective cell area. Immunohistochemistry for collagen IV and phospho-ERK was performed.10,11 For collagen IV detection, we used an antibody from Southern Biotech (1:500), and for phospho-ERK, from Santa Cruz (1:100). Ten different areas per organ (n=5 per group) were analyzed semiquantitatively. The data are expressed in arbitrary units (0 to 5), based on the staining intensity.

Western Blot
The following primary antibodies were used: polyclonal ERK1/2 (NEB; 1:1000), phospho-ERK1/2 (NEB; 1:1000), phospho-Elk-1 (NEB; 1:1000), phospho-JNK (Dianova; 1:1000), and p-EGFR (NEB; 1:1000). Peroxidase-conjugated secondary antibodies were from Sigma (1:5000). Blots were developed with the chemiluminescence substrate and visualized on Kodak films. Three to 5 cell stimulation experiments of each protocol were performed and quantified. For semiquantification, the most intense band was defined as 100%. All other bands of the experiment were calculated as percentage of the maximum.

p42/44 MAP Kinase Assay
The kinase assay for ERK 1/2 (p42/44 kinase assay) was performed with a kit from New England BioLabs. Briefly, after stimulation, active mitogen-activated protein (MAP) kinase was selectively immunoprecipitated. The precipitate was incubated with ATP and Elk-1 fusion protein in a kinase buffer. This allows active mitogen-activated protein (MAP) kinase was selectively immunoprecipitated. The precipitate was incubated with ATP and Elk-1 fusion protein in a kinase buffer. This allows active MAP kinase to phosphorylate Elk-1. Elk-1 phosphorylation was measured by Western blotting and quantified as described above.

Dichlorofluorescein to Measure Intracellular Reactive Oxygen Species
Intracellular reactive oxygen species (ROS) production was measured in rat VSMCs by the method of Ohba et al.11 Briefly, cells were kept in serum-free conditions for 24 hours (0.1% BSA). Cells were preincubated with Spi 10^-7 mol/L for 30 minutes or DMSO 10 μmol/L for 30 minutes. H2DCF-DA (2′,7′-dichlorofluorescein-diacetate, Sigma, 5 μmol/L) was added, and cells were stimulated with Ang II 10^-7 mol/L.

Statistics
Data are presented as mean±SEM. Statistical significance was tested by ANOVA, blood pressure and albuminuria by repeated-measures ANOVA and the Scheffé test, and ROS generation by SPSS. We used a general linear model with repeated measurements and post hoc by paired t test with the Bonferroni correction. A value of P<0.05 was considered statistically significant. The data were analyzed by use of StatView statistical software.

Results
MR Blockade Prevents Ang II–Induced End-Organ Damage In Vivo
Untreated dTGR (n=14) showed increased systolic blood pressure compared with Epl-treated dTGR (n=14) and nontransgenic (n=7) rats (204±5 versus 180±5 versus 119±6 mm Hg, P<0.05, respectively). Systolic blood pressure at week 7 was 61 mm Hg higher in Epl-treated dTGR compared with SD rats (P<0.05; Figure 1A). Urinary albumin excretion was markedly higher in dTGR than in SD rats: 17.8±2.1 versus 0.2±0.02 mg/d (P<0.001). Epl treatment reduced albuminuria (9.2±1.3 mg/d; P<0.05; Figure 1B). Epl also reduced collagen IV matrix deposition in the kidney (n=5 each; Figure 1C) and heart (data not shown). Untreated dTGR show ERK1/2 phosphorylation in the media of renal dTGR vessels, which was reduced by Epl (n=5 each; Figure 1D). Epl also reduced cardiac hypertrophy index (4.5±0.1 versus 5.4±0.2 mg/g; P<0.05; Figure 1E) and improved left ventricular diastolic function (normalized E/A ratio; Figure 1F) compared with untreated dTGR. Nevertheless, cardiac hypertrophy index remained increased in Epl-treated dTGR compared with nontransgenic SD rats (3.6±0.1 mg/g; P<0.05). These data document marked amelioration of renal and cardiovascular damage by MR blockade.

Ald Potentiates Ang II–Induced ERK Phosphorylation
Ang II (10^-7 mol/L) induced ERK phosphorylation in VSMCs with a maximal intensity after 2 minutes (Figure 2A). After Ald (10^-7 mol/L), the maximal intensity of ERK phosphorylation was observed at 10 minutes (Figure 2B). The combination of Ang II and Ald resulted in a stronger ERK phosphorylation at 1 and 2 minutes than with Ang II or Ald alone (Figure 2, C and E). Western blot and confocal microscopy experiments showed that Ang II and Ald at a lower concentration (both 10^-8 mol/L) still caused a similar potentiation (data not shown). Using the ERK1/2 MAP kinase assay, kinase activity was increased after Ang II and Ald alone as well as after the combination. However, the combination resulted in a higher MAP kinase activity, resulting in enhanced Elk-1 phosphorylation compared with the single compounds (Figure 2D). We also investigated Ang II and/or Ald-induced ERK phosphorylation in the presence of the protein synthesis inhibitors actinomycin D and cycloheximide. Neither inhibitor affected short-term ERK phosphorylation, supporting a nongenomic Ang II/Ald effect (data not shown).

Ald Potentiates Ang II–Induced JNK Phosphorylation
Ang II (10^-7 mol/L) also induced JNK phosphorylation with a maximal intensity after 2 minutes, whereas the maximal intensity of JNK phosphorylation after Ald (10^-7 mol/L) stimulation was observed at 10 minutes. When the cells were...
stimulated with the combination of both Ang II and Ald, a significantly stronger JNK phosphorylation was observed at 1 minute than with the single compounds alone (data not shown).

Ang II and Ald Signaling Is Mediated Through Oxygen Radicals
VSMCs were preincubated with GSH for 90 minutes before stimulation. GSH (2 mmol/L) preincubation suppressed both

Figure 1. A, Systolic blood pressure was lowered by Epl treatment (P<0.05) but not to Sprague-Dawley (SD) levels (mean±SEM). *P<0.05 dTGR vs dTGR+EPL; #P<0.05 dTGR+EPL and SD. B, Epl reduced albuminuria (P<0.001). C, Epl markedly reduced collagen IV deposition in glomerulus, basement membrane, and peritubular capillaries. D, Untreated dTGRs show p-ERK 1/2 immunoreaction in renal vessel media. Epl reduced p-ERK toward SD levels. E, Epl reduced cardiac hypertrophy. F, Epl restored E>A diastolic filling.
ERK (Figure 3, A through C) and JNK (Figure 3, D through F) phosphorylation after stimulation with Ang II (10⁻⁷ mol/L), Ald (10⁻⁷ mol/L), and the combination of Ang II and Ald. To verify the effect, we preincubated the cells with a distinct oxygen radical scavenger, Tiron. Tiron preincubation (10 μmol/L) caused a similar suppression of ERK phosphorylation induced by Ang II (10⁻⁷ mol/L), Ald (10⁻⁷ mol/L), and the combination of Ang II and Ald (data not shown).

**Effect of Spi on Ang II Signaling**

Next, we preincubated VSMCs for 30 minutes with Spi. Thereafter, the cells were stimulated with Ang II (10⁻⁷ mol/L) for 10 minutes. Spi decreased Ang II–induced ERK phosphorylation at 10 minutes but not at 2 minutes, as shown by Western blot (Figure 4A) and by confocal microscopy (Figure 4B). Consistent with this result, Ang II–induced EGFR phosphorylation was reduced by Spi at 10 minutes (Figure 4B; P=0.01); no effect was observed at 2 minutes. Furthermore, we measured the effect of Spi on ROS production induced by Ang II (10⁻⁷ mol/L) and Ald (10⁻⁷ mol/L). Spi reduced Ang II–induced ROS generation from 5 minutes onward (Figure 4C, P=0.05 at 5 minutes, 0.01 at 10 minutes).

The Ald (10⁻⁷ mol/L)–induced ERK phosphorylation was abolished with MR blockade (Figure 4D), whereas EGF (10 ng/mL)–induced ERK phosphorylation was not influenced by Spi (Figure 4E). Spi did not inhibit EGF-induced ROS production (data not shown).

**Ang II and Ald Signaling Is Mediated Through the EGFR**

We preincubated VSMCs with increasing concentrations (10, 100, and 300 nmol/L) of AG 1478, a specific EGFR blocker. We then stimulated the cells with Ang II (10⁻⁷ mol/L), Ald (10⁻⁷ mol/L), and a combination of both compounds. ERK phosphorylation was diminished dose-dependently by blocking the EGFR in all protocols (Figure 5, A through C).

**Discussion**

We found that Ang II and Ald both induced ERK 1/2 and JNK phosphorylation in VSMCs and that the agonists were additive. The stimulation was dependent on ROS generation, because GSH and Tiron strongly attenuated the phosphorylation. Spi did not inhibit early NADPH oxidase–dependent ROS generation. Instead, Spi affected the later phase of ROS...
generation, phosphorylation of the EGFR, and ERK 1/2 after Ang II stimulation. We then tested the notion that Ang II– and Ald-induced phosphorylation of ERK 1/2 was dependent on the EGFR. A specific EGFR blocker inhibited both the Ang II– and Ald-induced signaling events. We believe that these data are relevant to our in vivo findings that MR blockade with Epl reduced ERK 1/2 phosphorylation in dTGR vessels and greatly ameliorated Ang II–induced end-organ damage.

The MR is expressed not only in the cortical collecting duct but also in many other tissues, including the heart. Northern blotting, RNAse protection assay, RT-PCR, in situ hybridization, immunohistochemistry, and Ald binding studies have been performed in cardiac tissue. However, precise cellular localization studies have not been entirely satisfactory. Endothelial cells, cardiac fibroblasts, VSMCs, and cardiomyocytes have all been implicated in terms of MR expression. The MR can be occupied not only by Ald but also by glucocorticoids. As a matter of fact, the MR may dimerize with the glucocorticoid receptor. Brilla et al, and Young et al used different rat models and found that increased circulating Ald levels resulted in cardiac fibrosis. The DOCA-salt model results led to the suggestion that mineralocorticoid-mediated sodium entry into cardiac cells might be responsible. Further support came from the finding that Spi ameliorated the effects. Ang II regulates cardiac Ald production. Several studies were conducted to address the possibility that Ang II was responsible for cardiac fibrosis rather than Ald. Rocha et al showed...
Figure 4. A, Spi (preincubation for 30 minutes) reduced Ang II–induced ERK 1/2 phosphorylation and EGFR phosphorylation at 10 minutes ($P<0.01$; $n=4$ each) but not at 2 minutes. B, Same effects were documented with immunofluorescence and confocal microscopy. C, Spi (preincubation for 30 minutes) reduces Ang II–induced generation of ROS ($P<0.05$ from 5 minutes; $n=5$ each). D, Spi (preincubation for 30 minutes) abolished Ald–induced ERK 1/2 phosphorylation ($P<0.01$; $n=7$ each). E, Spi (preincubation for 30 minutes) did not influence EGF–induced ERK 1/2 phosphorylation ($P=0.88$; $n=3$ each).
that Ald infusion stimulates cardiac fibrosis in the rat. They suppressed Ang II production simultaneously with an ACE inhibitor. Benetos et al\textsuperscript{20} used a combined infusion of Spi and an ACE inhibitor in spontaneously hypertensive rats. They found that Spi reversed cardiac fibrosis. Earlier, we studied a similar rat model using Spi.\textsuperscript{21} In that study, we also found that Spi ameliorated cardiac hypertrophy and fibrosis, largely independently of blood pressure. Taken together, these studies support the notion that Ald induces cardiac fibrosis independently of other renin-angiotensin system components.

Ullian et al\textsuperscript{22} first suggested that Ald increases Ang II receptor number, increases Ang II–stimulated inositol phosphate responses, and prevents the Ang II–induced downregulation of Ang II receptors. This group also showed enhanced phospholipase Cγ–dependent signaling when VSMCs were preincubated with Ald for 24 hours before Ang II stimulation.\textsuperscript{23} In contrast to our findings, transcriptional regulation was involved in the studies by Ullian et al. We cannot exclude the possibility that genomic and nongenomic effects might have contributed to our in vivo observations. Nevertheless, the short duration of our in vitro experiments, as well as our results obtained with actinomycin D and cycloheximide, support a nongenomic effect.

Nongenomic effects have been reported for other steroids. Limbourg et al\textsuperscript{24} described a rapid and nontranscriptional activation of eNOS by corticosteroids that is transmitted via phosphatidylinositol 3-kinase and Akt. Nongenomic Ald-related effects have also been described in humans.\textsuperscript{6} For instance, Schmidt et al\textsuperscript{25} found that Ald, via nongenomic mechanisms, has diverse effects on the cardiovascular system that depend on the preexisting adrenergic state. Data from the rat remnant kidney model also support the idea that chronic Ald-related effects may include a nongenomic component. Greene et al\textsuperscript{26} studied 5/6 nephrectomy remnant kidney rats given AT\textsubscript{1} receptor blockers with ACE inhibitors and compared them with remnant kidney rats given these drugs along with Ald. In the former group, Ang II–related effects were blocked, and the rats were protected. In the latter group, given Ald, the effects were not ameliorated. This group resembled the no-treatment remnant kidney group. In their study, MR blockade did not reverse the effects of Ald, suggesting that MR-independent effects were present. The effects that we
observed appeared to be MR dependent, because Spi was capable of blocking the effects. In the case of the estrogen receptor, considerable evidence suggests that the receptor mediates both nuclear genomic and nonnuclear nongenomic effects.27,28 Exactly how nongenomic steroid-receptor signaling occurs is unclear.

Our data suggest that the MR interacts with Ang II–induced signaling, influencing ROS production, EGFR transactivation, and ERK phosphorylation. Ang II signals primarily via the AT1 receptor. The AT1 receptor is coupled to heterotrimeric G proteins, and stimulation results in the release of oxygen free radicals, phosphorylation of MAP kinases and receptor tyrosine kinases, protein kinase C activation, and activation of the transcription factors AP-1 and NF-κB.29 Ang II–induced ROS release has been suggested to function as a feed-forward mechanism.30 The early release depends on protein kinase C activation (H2O2; first peak at 30 seconds). The H2O2 activates src, which leads to EGFR activation. The activated EGFR mediates stimulation of PI3-K and the G-protein Rac. The latter binds to NADPH oxidase to activate generation of more O2− and H2O2, resulting in a sustained ROS generation that lasts up to 6 hours.30 Ald caused generation of ROS in VSMCs in vitro; however, this effect began later (6 to 8 minutes) than Ang II–induced ROS generation. This result suggests that Ald does not influence the early generation of Ang II–induced ROS production. The relevance of Ald-induced ROS production has also been shown in animal models. In aortic segments of Ald-infused rats, ROS levels were increased because of enhanced NADPH oxidase activity.31 Furthermore, rats receiving chronic Ald/salt treatment exhibited NADPH oxidase and NF-κB activation in their endothelial and inflammatory cells. The effect was ameliorated with MR blockade and antioxidants.32 In our study, both GSH and Tiron significantly inhibited Ang II/Ald signaling in VSMCs.

The EGFR also holds a key position in Ang II–induced signaling and is required for sustained Ang II–induced NADPH oxidase activation and ROS generation.30,33 Ald also mediated its effects via the EGFR. Ald enhanced EGF signaling, resulting in potentiated ERK 1/2 phosphorylation and Ca2+ homeostasis in MDCK cells.7 Further evidence about the role of the EGFR in Ald signaling comes from studies with CHO cells that lack the EGFR and do not respond to EGF or Ald. In EGFR-transfected CHO cells, EGF caused ERK 1/2 and src phosphorylation. Ald potentiated this signaling.8 From our results, we conclude that VSMCs require a functioning EGFR for Ang II– and Ald-induced tyrosine kinase signaling. The cytosolic tyrosine kinase c-src regulates trafficking of the EGFR out of the caveolae. This trafficking might be needed for EGFR internalization and transactivation.34 We did not investigate src-kinase phosphorylation. However, Ald potentiates Ang II–induced tyrosine phosphorylation in VSMCs (A. Fiebeler, unpublished data, 2003), and c-src may be one of these tyrosine kinases. Altogether, the feed-forward model,30 as well as our findings, suggests that the interaction between Ang II and Ald is downstream of the first Ang II–induced ROS production but upstream of the EGFR. Kinases such as src and their regulating phosphatases may well be an interconnection between the 2 signaling pathways.35

Our earlier studies in dTGR indicated that the NADPH oxidase is strongly activated in this model.11 Both NF-κB and AP-1 are activated, and both control the inducible expression of genes whose products are part of the inflammatory response. The JNK and ERK pathways are 2 members of the MAP kinase family that are also activated by ROS. The ERK pathway also modulates the expression of genes via phosphorylation of the transcription factor Elk-1, which controls the production of the c-Fos transcription factor. Nevertheless, not all Ang II–induced MAP kinase activation is under control of the EGFR.36 Our data show that not only Ang II but also Ald participate in both JNK and ERK signaling. They suggest that blockade of both the AT1 and the MR receptor may be necessary to accrue maximal effects in terms of vascular protection.

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